MICROBIOLOGICAL REVIEWS, Dec. 1987, p. 419–430
0146-0749/87/040419-12$02.00/0

Vol. 51, No. 4

Regulation of Early Adenovirus Gene Expression

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INTRODUCTION

Cellular phenotype is determined by the specific set of gene products unique to a given cell. Since all cells in an organism bear the identical set of genes (with some exceptions such as the antibody-producing cells), the control of cellular phenotype is a manifestation of the control of expression of the genes in a particular cell. An understanding of the underlying mechanisms of gene control, including the molecules that participate in gene control, is of obvious importance to attaining a real understanding of the basis for cellular differentiation, early embryonic development, and oncogenesis, to name but a few examples.

The use of animal viruses has proved invaluable in the elucidation of these mechanisms. Viruses offer the advantage of simple gene systems that are easy to manipulate in the context of their normal control; they afford the opportunity to analyze high-copy genes by increasing the multiplicity of infection; and, probably most important, they provide a genetic basis for defining functions involved in the regulation of gene expression. In no other case have these advantages been better exemplified than in that of adenovirus. The study of adenovirus gene structure and gene regulation over the years has provided many of the underlying principles on which present day experiments are built. The definition that the start site of polymerase II transcription specified the messenger ribonucleic acid (mRNA)5′ end (156), the discovery of RNA splicing (6, 18), the finding that RNA cleavage rather than transcription termination generated the mRNA 3′ end (99), and the development of systems for in vitro transcription (86, 143) that have allowed the isolation of transcription factors were all first realized in adenovirus systems. Since these initial findings, the study of adenovirus gene expression has rapidly progressed. The focus of this review is the regulation of expression of the early genes of the virus. For more general considerations, the reader is referred to several recent reviews of the subject of adenovirus gene structure or virus replication in general (115, 128, 132).

GENE ORGANIZATION

The structure and genome map locations of the early viral RNAs have been defined in a number of ways, including electron microscopy (17, 70), S1 nuclease mapping (9), in vitro translation of hybrid-selected RNA (81, 125), and complementary deoxyribonucleic acid (DNA) cloning (105, 122). Many of the details of this mapping have been reviewed previously (115, 132, 139, 140) and are summarized in Fig. 1. Although function has been assigned to many of the products of the early RNA, there remain many species for which there is no known activity.

Although there are numerous reasons for defining the structure of a given gene, this information is crucial for allowing an understanding of the regulation of expression of the gene. As discussed in detail elsewhere (98), the process of formation of a eucaryotic mRNA is complex, involving not only transcription but also a variety of RNA processing events before the mature mRNA is produced. Thus, if the steady-state concentration of a particular viral mRNA varies from one condition to another, it could be the result of an alteration of any one of the preceding events in mRNA biogenesis. This complexity is best illustrated by examining the organization of early adenovirus transcripts as depicted in Fig. 1. The early viral transcripts map throughout the viral genome, encoded by both DNA strands, and are organized into six distinct transcription units (a transcription unit being defined as that section of a genome between a promoter and a terminator for RNA polymerase). The definition of these transcription units was accomplished by both ultraviolet mapping (8, 145) and nascent chain analysis (26). Three of the early transcription units (major late [ML], E2, and E3) are complex since they contain multiple polyadenylic acid [poly(A)] addition sites, and each of the six transcription units produce multiple RNAs by alternative splicing. Therefore, the steady-state level of one of the early RNAs could be governed by complex events. For instance, the level of an

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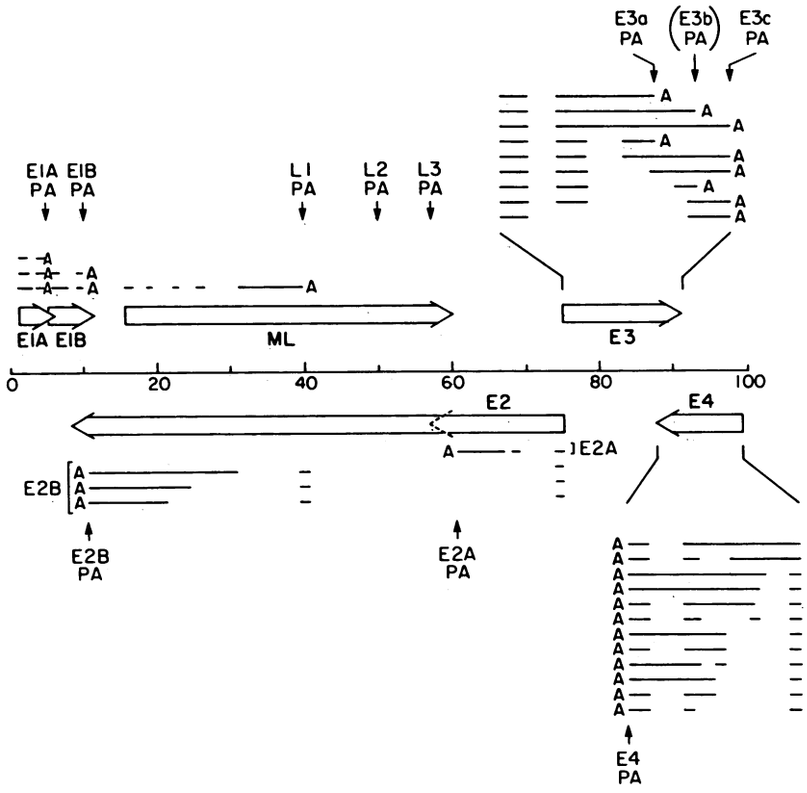

FIG. 1. Map of early adenovirus transcription units and major species of mRNAs encoded by each. Transcription units are depicted as the open arrows. The structure and map positions of the major species of early RNAs are shown as the broken lines. For details of the mapping, see references 2, 9, 17, 70, 105, 122, 139, and 140. Sites for poly(A) addition (mRNA 3' ends) are indicated by PA. The E3b site is tentative as no complementary DNA has been isolated with this 3' end.

E2 product could result from control of transcription initiation, poly(A) site choice (E2A or E2B), or splicing choice.

TEMPORAL EXPRESSION OF EARLY GENES

The pattern of early viral gene expression has been determined by a variety of assays, although the initial descriptions measured protein synthesis. Even from these early studies, it was clear that there was control of early gene expression since the kinetics of synthesis of various early proteins differed (95). A further analysis of early gene expression indicated that most of the control of appearance and decline of early gene products was the result of transcriptional regulation (100). The transcription rates from each of the early regions varied with respect to both the onset of activity and the maintenance of transcription rates. In particular, the E1A and E4 transcription units are activated very early in the infection, followed by E3, E1B, and finally E2. Transcription of E1A and E1B continues well into the late phase of infection, whereas transcription of E4 is rapidly shut off and transcription of E2 and E3 slowly declines. These kinetics are generally coincident with the appearance of the gene products during the course of an infection.

Although there is no evidence for post-transcriptional control of early gene expression, there is clear evidence in three cases for such control when early regions are expressed both early and late in infection. First, a 9S E1A mRNA is produced late in infection but not early, whereas the 13S and 12S E1A RNAs are produced both early and late (121). All three RNAs derive from a common precursor with a single poly(A) site, suggesting that there is a change in splicing control (105). Second, the E1B 20S mRNA is the predominant species early in infection, whereas late in infection the 14S E1B mRNA becomes the major RNA. In this instance, it appears that differential stabilities of the RNAs are responsible for the change in ratio (144). Finally, the proximal half of the major late transcription is expressed both early and late (3, 17, 102, 117). The major product during early infection is a single L1 mRNA (3, 102), whereas the same region expressed late in infection produces at least three L1 mRNAs, five L2 mRNAs, and three L3 mRNAs (115). The families of L1, L2, and L3 RNAs are determined by distinct poly(A) sites, and the individual species within a family are defined by a distinct splicing event (99). Therefore, changes in both poly(A) site choice and splicing choice appear to be responsible for the observed changes between early and late infection. In the case of L1 RNA production, pulse-labeling studies demonstrated that a change in splicing is almost certainly responsible for the shift in RNA production (102).

The basis for these changes remains obscure. On a gross level, it appears that late gene expression can only derive from replicated templates since a parental unreplicated genome in a late infected cell does not express late gene products (131). However, it is not absolutely clear whether a low level of late gene products from the unreplicated templates would have been detected under these conditions.

Certainly, no viral gene products have been identified which might mediate these changes, although it is interesting that changes in RNA splicing patterns have been found in cells infected with a mutant in the VA gene (130). The VA RNA itself does not appear to direct these changes, but more likely this is the result of the lack of synthesis of a late viral protein. Although the analysis of in vitro systems for splicing and, more recently, polyadenylation are beginning to define factors necessary for the processing reactions (85, 116), no information is yet available concerning the basis for alternative choices in RNA processing pathways.

The tight control of early gene expression directed by transcription rate changes is possible since the early mRNAs decay rapidly, with half-lives of no more than 20 min (146). The product of the E2A region, the 72-kilodalton (kDa) DNA binding protein, appears to mediate the rapid decay of early mRNA (4). In the absence of a functional E2A gene product, there is an overproduction of each viral mRNA (14). Direct assays of turnover rates demonstrated that the stability of the early RNAs increased three to fivefold when produced in a *ts*125 infection, a mutant encoding a thermolabile 72-kDa protein (38, 135). Although the 72-kDa protein does appear to interact with mRNA inside the cell (19; I. Lazaridis, A. Babich, and J. Nevins, submitted for publication), such interaction does not appear to target RNAs for destruction since there is equal association with viral and cellular RNAs (Lazaridis et al., submitted). There is no evidence that this 72-kDa protein-directed turnover is a regulatory event other than ensuring that transcriptional control will result in rapid changes in the mRNA population due to the short half-life of the RNA.

TRANSCRIPTION CONTROL

As suggested in the preceding section, the activation mechanisms of the various early transcription units are not identical, suggesting an underlying control of this process. Indeed, through the analysis of adenovirus mutants defective in the E1A function (47, 61), it was established that production of the early RNAs depended on the E1A gene (7, 62). Soon thereafter it was shown that the positive control of E1A was largely transcriptional (96), although there appeared to be post-transcriptional effects as well (66). Although it has not been demonstrated in the context of control of viral genes, E1A also appears to exert negative effects on transcription as well as the positive control (11, 138). Possibly this is for the purpose of autoregulation in the context of a productive infection (120), but it may also be involved in the shutoff of E2 transcription (43). Finally, in addition to the regulation of transcription by E1A, there is negative transcription control mediated by the E2A product. As mentioned previously, transcription of the E4 region quickly reaches a peak level and then declines. It was initially observed that this decline required protein synthesis, suggesting an active repression, possibly by a viral protein (100). Indeed, it was found that the product of the E2A gene, the 72-kDa DNA-binding protein, was required for this shutoff (101). Later experiments demonstrated a specific repression of E4 transcription in vitro by the purified 72-kDa protein (44), although the mechanistic basis for the repression is yet to be understood since there is no evidence for sequence-specific binding by the 72-kDa protein (29).

ACTIVATION OF TRANSCRIPTION BY E1A

Since the initial discovery of the regulatory role of E1A, a great deal of effort has been devoted to understanding the mechanism for E1A-mediated control. Several features contribute to the attractiveness of this particular system of gene control. First, it represents a system of coordinate gene control whereby six viral promoters (E1A, E1B, E2, E3, E4, and ML) as well as several cellular promoters (details discussed below) are subject to activation by a single regulatory gene product. Second, the system is sufficiently simple that one may hope to understand how an entire regulatory circuit operates. Finally, it has become clear from a number of studies that E1A mediates transcription control through cellular components, and thus an understanding of the activation of these viral genes may lead to an understanding of transcription control within the cell.

The "E1A gene" is actually a set of three transcripts that encode three separate proteins (Fig. 2). As indicated above, the 9S mRNA is only produced in cells infected late (121), and therefore the product of this RNA is not involved in transcription control. The 13S and 12S RNAs, which are expressed early, encode proteins of 289 and 243 amino acids, respectively, based on the complementary DNA and genomic DNA sequence (105, 136). These two proteins are identical in sequence except for 46 additional amino acids near the middle of the 13S product. Studies of various E1A mutants have demonstrated that the 289-amino acid 13S RNA product is the primary activator of early viral transcription (39, 90, 92, 108), although it does appear that the 12S product can effect an inefficient stimulation of the same genes stimulated by the 13S product (28, 78, 147). As reviewed recently, three regions within the E1A gene exhibit sequence conservation among virus strains, and various mutational studies have suggested that these regions closely correspond to functional domains (67, 91).

Experiments with cycloheximide or various other protein synthesis inhibitors demonstrated an increase in early viral RNA production in the absence of protein synthesis, suggesting a negatively acting regulatory component in the

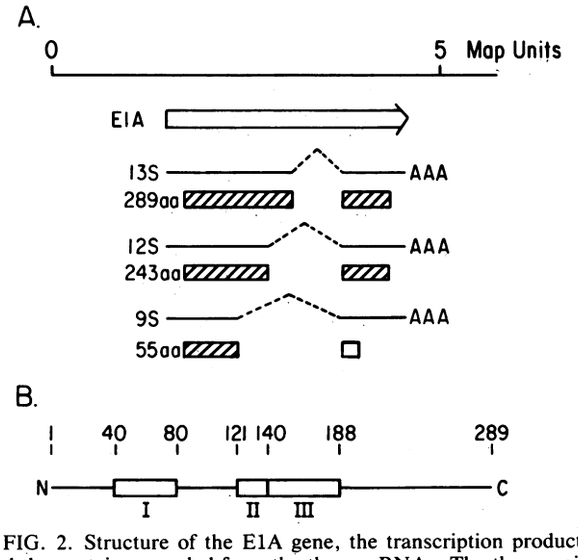

FIG. 2. Structure of the E1A gene, the transcription products, and the proteins encoded from the three mRNAs. The three major E1A RNAs, 13S, 12S, and 9S, are depicted together with a schematic of the proteins they encode. The C-terminal half of the 9S product derives from a reading frame distinct from that of the other sequences. Shown at the bottom is a representation of the sequences conserved among various adenovirus serotypes (67). aa, Amino acids.

infection (21, 24, 104). The involvement of negatively acting cellular components in E1A-mediated induction was inferred from initial experiments that demonstrated that the inhibition of protein synthesis could in part relieve the requirement for E1A (66, 96). In addition, further analysis of the effect of the inhibition of protein synthesis in the presence of E1A indicated that at least part of the effect was due to an increase in transcription of the early viral genes (22). These findings led to the suggestion that the basis for E1A stimulation of transcription involved the relief of negative control imposed by the cell. Even though further experiments have indicated that such negative control does not appear to involve a repressor, any activity which reversed the effect of E1A (for instance, inactivation of a positively acting transcription factor) would fit this role.

The indirect nature of E1A *trans* activation was underscored dramatically by the observation that other viral *trans*-activating genes could replace E1A in the activation of early adenovirus transcription. This was initially demonstrated with pseudorabies virus, a member of the herpesvirus family, but it has now been found that a number of other viral *trans* activators can do the same. Coinfection of cells with the E1A deletion mutant *dl*312 and pseudorabies virus resulted in activation of early adenovirus transcription: in fact, more efficiently than in a wild-type adenovirus infection (27). This activation did not occur in cells infected with a temperature-sensitive mutant of pseudorabies virus deficient in immediate early (IE) gene function. Furthermore, transfection assays utilizing the adenovirus E2 promoter and a cloned copy of the pseudorabies immediate early gene demonstrated that the herpesvirus *trans* activator could indeed stimulate the adenovirus promoter and could do so as well as, if not better than, the E1A gene (56). Thus, a heterologous activator (IE), from a virus with no genetic relationship to adenovirus was as effective as the homologous activator (E1A) in the stimulation of adenovirus transcription. These results argued strongly that the action of E1A was not direct in the sense of a DNA-binding protein with specificity for each of the early adenovirus promoters. In support of this is the repeated failure to detect DNA binding activity with the E1A protein (28), although one report has suggested that the E1A protein could interact with DNA in the presence of cell extracts (71).

Analysis of early gene expression in *dl*312 infections has provided evidence for a cellular regulatory activity similar in nature to E1A. Imperiale et al. (58) found that certain cell lines could partially complement the E1A defect in *dl*312 for early transcription whereas in other cells there was an absolute requirement for the viral E1A. Possibly the most informative example was the mouse F9 teratocarcinoma cell line, a tumor cell line with properties characteristic of the undifferentiated early embryo (87, 126). The cells in culture are rapidly growing undifferentiated cells that form tumors when injected into animals. In the presence of retinoic acid and cyclic adenosine 5′-monophosphate, the cells differentiate and acquire properties similar to that of primitive endoderm and lose their proliferative and oncogenic capacity (127). It was found that F9 cells could complement an E1A mutant for early gene expression whereas the differentiated F9 cells could not (58). Thus, there appeared to be a cellular activity, similar in function to that of the viral E1A gene, which was controlled during differentiation.

The possibility that E1A control involved cellular components and factors was consistent with the previous finding that the genes subject to E1A control were not limited to the set of early genes on the viral chromosome. Initially it was found that adenovirus infection induced the synthesis of a 70-kDa cellular protein that turned out to be the major heat shock protein (97). This induction required E1A and, with the use of a complementary DNA, was shown to be transcriptional (65). Furthermore, the kinetics of activation of hsp70 transcription were identical to that of the early viral genes. Subsequently, it was shown that the β-tubulin gene was activated by E1A in a lytic infection (123). More recently, several other genes have been found to be induced by E1A. An additional heat shock gene, hsp89, is activated by E1A during lytic infection (118). Furthermore, the synthesis of a cellular protein termed PCNA (proliferating cell nuclear antigen), also known as cyclin, is stimulated in an adenovirus infection, dependent on the E1A gene, as is the gene encoding thymidylate synthetase (155). Although it is not yet clear that the increase in PCNA synthesis is due to an increase in transcription, the induction shares an interesting property with that of hsp70. In contrast to the activation of the early viral genes, the induction of hsp70 (118) and PCNA (155) can be mediated by the 12S E1A gene product as well as the 13S product. Interestingly, recent data have shown that PCNA is a factor required for the in vitro elongation of simian virus 40 (SV40) DNA synthesis in cell-free extracts (106). Perhaps the E1A-mediated induction of PCNA is part of the process of the induction of DNA synthesis of E1A, a possibility consistent with the observation that the E1A-regulated hsp70 gene is also cell cycle regulated (64, 148). Finally, in line with the role of E1A in induction of cellular proliferation is the observation that several cell cycle-regulated genes appear to be induced by adenovirus infection, although it is not clear that this is a function of E1A (83).

In addition to the activation of these chromosomally located cellular genes, it was found that a multitude of plasmid-borne genes could be activated by E1A in transfection experiments. Transfection assays demonstrated that the human β-globin promoter was stimulated by E1A as well as by the pseudorabies virus immediate early gene (42), as was the early SV40 promoter in these experiments, but the α-globin promoter was only weakly stimulated, if at all. Various other experiments have now shown a number of promoters to be responsive to E1A or herpesvirus regulatory genes, either in transient transfection assays or when the target gene is incorporated into either the adenovirus or the cellular chromosome (23, 35, 82, 129). Thus, the effects of E1A in stimulating transcription appeared to be widespread and certainly not restricted to the set of viral genes that are the normal targets of activation. Furthermore, this phenomenon appears to be typical among a variety of viral *trans* activators that have in common the property that they encode nucleus-localized proteins which are oncogenic (for review, see reference 68).

Finally, it has been shown that the genes subject to E1A activation are not limited to those transcribed by RNA polymerase II since E1A appears to influence the expression of genes transcribed by RNA polymerase III. Extracts prepared from adenovirus-infected cells were found to be more active in the transcription of the VA gene than extracts from uninfected cells (34, 53). This increased activity did not occur in cells infected with E1A mutants. Furthermore, cotransfection assays demonstrated that the E1A gene alone could effect a stimulation of a variety of promoters recognized by polymerase III (5, 34, 53). In contrast to the induction of polymerase II transcription, the induction of a cellular chromosomal gene transcribed by polymerase III has yet to be shown. Regardless, the importance is the

demonstration that E1A has the capacity to stimulate polymerase III transcription.

Mechanism for E1A Action

A number of studies have analyzed the sequence requirements in various E1A-inducible promoters for both basal transcriptional activity and induction by E1A. Essential sequences in each of the early viral promoters, including E1A (48, 49, 111), E1B (13, 150), E2 (25, 57, 59, 60, 69, 94, 154), E3 (77, 142), E4 (36, 37, 45, 76), and major late (52, 55, 63, 80, 84, 88, 153), have been mapped in some detail. In addition, the critical sequences in the hsp70 promoter for E1A control have been delineated (M. C. Simon, T. Fishe, B. Benecke, N. Heintz, and J. R. Nevins, manuscript in preparation). From these data, several conclusions can be drawn. First, there is no one sequence conserved in the essential regions of the early viral promoters. Thus, consistent with the analyses described above, there is no firm basis for a direct role for E1A in coordinate transcription control. Second, although there is no one single sequence found in each promoter, there are sequences found in more than one promoter, suggesting the use of common factors by the various promoters. Third, for the most part the sequences important for uninduced transcription ($E1A^-$) are also those required for induced transcription ($E1A^+$). There are exceptions. An E4 promoter deleted of sequences upstream of −158 still functions in the absence of E1A at a level equivalent to a promoter retaining these sequences (36). However, the induced transcription from this deleted promoter is greatly reduced. Likewise, a linker-scanning mutation in the E1B TATA sequence appears to have little effect on basal transcription but does impair induced transcription (150). From these studies, one must conclude that in some cases (E4 and E1B) induction may involve a specific factor not used for basal transcription whereas in other cases the same factor(s) necessary for uninduced is also required for induced transcription.

Based on long-term infections with an E1A mutant, Gaynor and Berk (33) suggested that the action of E1A stimulated the formation of stable complexes on early viral promoters. Although an E1A mutant was inactive in early transcription at 8 h postinfection, there was substantial transcription at 33 h. However, a second E1A mutant introduced into cells at this time underwent the same slow activation process, thus demonstrating a *cis* effect. A direct measure of this was obtained by an in vivo exonuclease III footprint analysis comparing complexes on the E2 promoter in wild-type virus- and *dl*312-infected cells. A complex was detected in the wild-type virus-infected cells at a position on the E2 promoter coincident with previously defined critical sequences (72). However, no such complex was detected in the *dl*312 infection, although if the *dl*312 infection was allowed to proceed for 30 h, there was the appearance of a complex. It appeared that proteins associated with the E2 promoter and did so more rapidly in the presence of the E1A gene product. These results are also consistent with the observation that there was an increased number of templates transcribed in extracts from adenovirus-infected cells compared with uninfected cells and that this was dependent upon the E1A gene (79). Therefore, from several points of view, it appears that E1A accelerates the formation of stable promoter complexes.

E2F Factor

If E1A action involves stimulation of formation of stable promoter complexes, as suggested by the E1A mutant experiment of Gaynor and Berk (33) and in vivo exonuclease III analysis (72), then it follows that there must likely be an alteration of one or more key proteins that trigger this association. Recent experiments have provided evidence for the existence of such a protein. Utilizing the gel mobility shift assay (30, 32), Kovesdi et al. (73) detected a protein that specifically recognized the adenovirus E2 promoter. The protein bound to sequences within the promoter defined as important by deletion mutagenesis and also within the domain defined by the in vivo mapping experiments. Of most significance was the finding that there was a large increase in the level of the factor, as measured by DNA binding, in extracts of infected cells versus uninfected cells. Furthermore, this increase depended on a functional E1A gene. Thus, this cellular factor, termed E2F, exhibited the properties expected of a factor mediating E1A stimulation of this E2 promoter.

Additional experiments have established a firm relationship between the level of E2F in cell extracts and the transcription of the E2 promoter inside cells. For instance, the kinetics of appearance of E2F during an early infection coincide with the activation of E2 transcription (R. Reichel, I. Kovesdi, and J. Nevins, Proc. Natl. Acad. Sci. USA, in press). In addition, the activation of E2F requires the 13S product of the E1A gene, also a requirement for induction of E2 transcription. As described above, previous work has demonstrated the existence of a cellular E1A-like activity based on the ability to partially complement the transcriptional inducing defect of an E1A mutant (58). Particularly informative was an analysis of the mouse F9 teratocarcinoma cell line since undifferentiated F9 cells exhibited an E1A-like activity which disappeared upon induction of differentiation by retinoic acid and cyclic adenosine 5′-monophosphate. Examination of extracts of F9 cells and differentiated F9 cells revealed a coregulation of E2F (107). There was a high level of E2F in F9 extracts, whereas the extracts of differentiated cells were devoid of the factor. However, if the viral E1A gene was introduced into the differentiated cells by means of a viral infection, the E2F factor reappeared. Thus, from a number of different lines of study, there exists a close correlation between the level of E2F and the presence of an E1A activity, either cellular or viral.

In addition to these correlations, there is direct evidence for a role of E2F in stimulating transcription. A DNA fragment containing a single E2F-binding site could confer increased transcriptional activity to the mouse β-globin promoter when inserted just upstream of the promoter (74). This increase in transcription required the E1A protein, consistent with the fact that E1A increases the level of E2F. Also, that the binding of E2F was actually important was established by first treating the plasmid with *Hha*I methylase prior to transfection. The E2F-binding site (TT TCGCGC) is a substrate for *Hha*I methylase and, as a result of the methylation, binding of E2F is inhibited. Methylation also inhibits the ability of E1A to stimulate the β-globin fusion, demonstrating that E1A indeed stimulates via the E2F factor. It therefore appears certain that the E1A-mediated control of E2F is responsible for the stimulation of E2 transcription.

Other experiments have defined the relative role of E2F in E2 transcription. There are two binding sites for E2F within the E2 promoter (Fig. 3): one located between −33 and −49 and the other at −53 and −71 and each containing the sequence TTTCGCGC (151a). However, mutagenesis of the E2 promoter has shown that sequences between −71 and

FIG. 3. Structure of the E2 promoter and location of protein factor-binding sites. Depicted at the top are the sequence elements in the E2 promoter that are involved in protein binding. The domains of protein interaction, as defined by deoxyribonuclease footprinting and methylation interference (10, 73, 119, 151a), are shown below. Also shown is the position of a block to exonuclease III digestion observed in vivo which presumably reflects a promoter-bound complex (72).

−80 are important for promoter activity (59, 94), suggesting that at least one other factor might interact with the promoter. Indeed, gel shift assays detected a factor binding to these sequences (119). Furthermore, direct footprint analysis with crude nuclear extracts demonstrated that both factors, E2F and the upstream factor, could interact simultaneously (151a). However, unlike E2F, the level of the upstream factor does not fluctuate with respect to E1A (119, 151a). Therefore, both factors appear to be required for full promoter activity but E2F appears to be the limiting component. This finding is of interest when the analysis of in vivo protein-DNA interaction is considered. In the absence of E1A function, the E2 promoter was void of any stable complex (72). Thus, despite the presence of the EPF factor, there is apparently no stable interaction with the promoter. One might speculate that the formation of the stable promoter complex requires the presence of E2F and such complexes only form when it rises to a sufficient level.

Basis for Control of E2F by E1A

One could imagine that E1A could increase the actual amount of the E2F factor in the cell or, alternatively, E1A might simply activate the binding capacity of a dormant pool of E2F. The assays for E2F induction involved simple DNA binding and thus do not discriminate between these two possibilities. A resolution to this question has been obtained by measuring E2F levels after adenovirus infection in which cycloheximide was added, to determine whether the increase in E2F levels requires the synthesis of new protein. This is a somewhat difficult experiment since cycloheximide added at the time of infection would of course also prevent formation of the E1A protein. However, if cycloheximide is added after 1 h of infection, sufficient E1A protein is produced, and it was found that there was little or no difference in the level of E2F plus or minus cycloheximide (Reichel et al., in press). Therefore, it appears that the induction of E2F is posttranslational, and very likely E1A must mediate an alteration of a preexisting protein to effect an increase in binding activity. The exact nature of the alteration is not clear at the moment nor is it clear if the alteration is mediated by the E1A protein itself. For instance, it is possible that E1A initiates a chain of events that results in the modification of the E2F factor. If the activation is direct, it may be possible to demonstrate the presence of E2F among the cellular proteins immunoprecipitated with E1A (46, 151). Possibly, the purification of the factor, the production of an antibody, and the subsequent analysis of the protein will lead to an understanding of the basis of the control. There has been progress towards this goal as the E2F factor has now been purified to near homogeneity. It appears to be a protein of 54,000 molecular weight and is a relatively scarce protein within the cell; there are probably no more than 1,000 copies of active factor in a virus-infected cell (A. Yee and J. Nevins, manuscript in preparation).

Coordinate Control of Transcription

In addition to the E2 promoter, the E1B, E3, E4, and ML promoters are also stimulated by E1A. Furthermore, E1A transcription is reduced three to fourfold in the absence of a functional E1A protein, indicating that there is stimulation of the E1A promoter as well (96). Finally, as described above, at least three cellular promoters, hsp70, β-tubulin, and hsp89, are stimulated by E1A during a lytic viral infection, and many more have been shown to be activated by E1A in transfection assays. Therefore, given that E2F mediates stimulation of E2 transcription, one can question whether this factor plays a role in transcription of the other inducible promoters. Competition assays for E2F binding indicated that it does bind to the E1A regulatory sequences but not to any of the other promoters regulated by E1A protein (74). There are two binding sites for E2F within the upstream region of E1A previously defined as the E1A enhancer. Assays of deletion mutants of this region are consistent with these binding sites being important for E1A transcription (48, 49), indicating that the E2F factor may be critical for stimulation of both E1A and E2 genes. However, due to the lack of binding to the other promoters, this factor cannot be involved in the transcription of any of the other inducible genes. One must conclude that E1A control involves multiple promoter-specific factors.

If the E2F factor is not involved in stimulation of the other inducible promoters, what are the critical factors and what is the basis for coordinate control? Various studies have identified proteins that interact with the E1B promoter (150), the ML promoter (15, 89, 112), the E4 promoter (82), and the hsp70 promoter (93, 149). Certainly, the best studied of these is the factor which interacts with the upstream regulatory site of the ML promoter and which has been purified to near homogeneity and shown to stimulate transcription in vitro (16). However, there is no evidence to suggest that any of these proteins are involved in E1A stimulation of the respective promoters. They undoubtedly are essential for transcription but likely are not regulatory. Although there is as yet no direct evidence, several lines of study have implicated a TATA binding factor in E1A-mediated stimulation of transcription of certain promoters. The initial assays of E1A induction of the β-globin promoter demonstrated that only a TATA element was necessary for E1A stimulation in transfection assays and that a point mutation in the TATA sequence abolished the response to E1A (42). More recently, Berk and colleagues, utilizing promoter mutations engineered into adenoviruses, have demonstrated that sequences upstream of the adenovirus E1B TATA could be deleted and yet inducibility by E1A was retained whereas a linker-scanning mutation directed at the TATA element eliminated E1A inducibility without affecting the basal level of promoter activity (150). Thus, the promoter appeared to still function but was not responsive to E1A. Interestingly, the same may be true for the hsp70 promoter with respect to E1A induction. It appears from an analysis of a series of deletion mutants as well as internal substitution mutants that the critical element for E1A control is the TATA sequence (Simon et al., in preparation).

Although not involving E1A but rather the pseudorabies virus immediate early gene, a biochemical approach has come to the same general conclusion. Extracts prepared from cells infected with pseudorabies virus could stimulate transcription in vitro when mixed with a HeLa nuclear extract (1). The stimulation only required the TATA element. Furthermore, recent data suggest that the stimulation is dependent on the pseudorabies virus immediate early protein and is mediated through the TFIID transcription factor (S. Abmayr and R. Roeder, manuscript in preparation), the factor that recognizes the TATA element (103, 112). In addition, since the activation can occur in vitro, it would appear not to involve the synthesis of additional TFIID molecules (Abmayr and Roeder, in preparation). Finally, assays of an extensive set of thymidine kinase promoter mutations have suggested that the TATA sequence is the more important element for *trans* activation by the herpesvirus immediate early gene ICP4 (20). Although these studies do not involve E1A, given the similarities in action of the two regulatory proteins it seems likely that similar mechanisms will apply to both.

In addition to the stimulation of polymerase II transcription, E1A also stimulates polymerase III transcription (5, 34, 53). Analysis of extracts indicated that the induction of transcription from promoters utilizing polymerase III may involve the TFIIIC factor (53, 152), the factor which recognizes the internal promoter element of class III genes (31, 75, 114). From recent experiments, it appears that this may involve a modification of a preexisting TFIIIC that increases the stability of its interaction with the promoter (W. Hoeffler and R. Roeder, manuscript in preparation).

Based on all of these recent data, it would appear that E1A can affect the activity of at least three distinct DNA-binding proteins (E2F, TFIID, and TFIIIC) and by so doing cause a stimulation of transcription. One must then ask what these apparently unrelated protein factors share in common to allow for coordinate control by E1A. Possibly important is the observation that at least in two cases, if not all three, the induction likely involves a post-translational modification or alteration. Given this, we might speculate that, although these proteins must recognize different DNA sequences, they may share a common domain that is the site of action of E1A. The E2F, TFIID, and TFIIIC factors may all possess a common domain which is recognized by E1A (Fig. 4). Thus, a group of distinct factors are commonly recognized by the regulatory protein. Upon the action of E1A, each is converted to an active form. In the case of E2F, the activation results in an increased binding capacity. In the case of the other factors, the step of activation is not clear and may not involve an activation of DNA binding but rather an activation of function independent of binding. In either case, the critical step would be the concerted action of E1A.

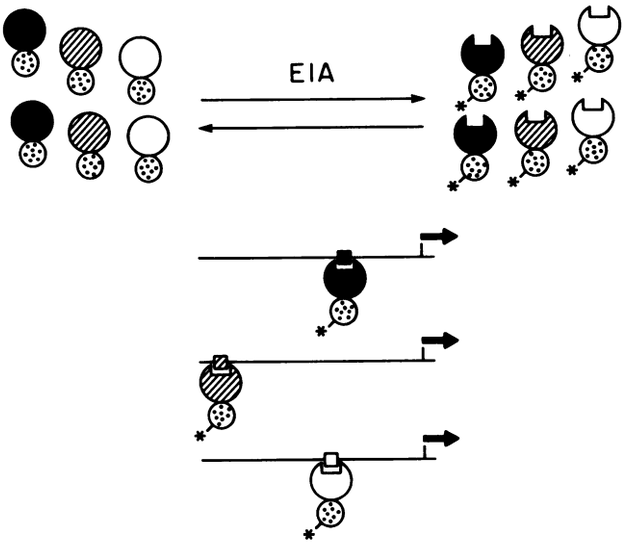

FIG. 4. Possible mechanism for coordinate control of transcription by E1A. Depicted are a set of three transcription factors, each with distinct DNA binding domains (solid, hatched, or open circles) but common regulatory domains (stippled circles). Upon the action of E1A, the regulatory domain is modified (asterisk), which induces a conformational change allowing the factor to bind to its DNA recognition site.

REPRESSION OF TRANSCRIPTION BY E1A

In addition to the positive activation of transcription mediated by E1A, certain transcription regulatory elements are negatively controlled by E1A. Chambon and colleagues (11) initially demonstrated an E1A-mediated repression of the enhancers of SV40, polyomavirus, and E1A. Very similar observations were made by Velcich and Ziff (138), and now several other regulatory elements, including enhancers from cellular genes, have been shown to be targets for negative action by E1A (124, 137). The molecular basis for the negative effect is not understood, although it does appear to involve a repressor (negatively acting DNA binding protein) in the sense that the negative effect on the SV40 enhancer could be eliminated by introducing increasing amounts of the enhancer fragment (11). However, whether the effect is direct or indirect with respect to the action of the E1A protein is not settled nor is there any information concerning the target sequence within the various enhancers for the repression. That the effect may be indirect is suggested by experiments that demonstrate that the immunoglobulin heavy-chain enhancer responds to E1A in either a positive or a negative manner depending on the cell type. In a lymphoid cell, E1A represses transcription of the heavy-chain locus (50), whereas if the heavy-chain gene is introduced stably into a fibroblast, it now is activated by E1A (12). Whether these effects are the result of different substrates (positive or negative factor) available for the action of E1A or result from a direct interaction of E1A with the DNA, influenced by the presence of other DNA-binding proteins, is not clear.

In contrast to the activation of viral transcription, the 12S product appears to be as effective as the 13S product in the repression of transcription (11, 92, 138). Indeed, site-directed mutagenesis of the E1A gene has defined the second conserved domain (Fig. 2) as critical for the repression activity (82), whereas region three of E1A appears to be critical for the activation of early viral transcription.

A series of observations from the mouse F9 teratocarcinoma system has led to speculation that negative control by a cellular E1A activity is important in the differentiation process. As detailed before, F9 cells contain an E1A-like activity which disappears upon differentiation (58). It is also known that the viral E1A can negatively regulate the SV40 and polyomavirus enhancers. This fact together with the previous observation that both polyomavirus and SV40 are inactive in F9 cells but are expressed in the differentiated cells has led to the suggestion that the inactivity of these viruses in the F9 cells was due to a repression by the cellular

E1A-like activity. Recent experiments have indeed demonstrated that at least part of the control in F9 cells appears to be a repression (40). Given these results, one wonders how general such effects may be with respect to the activation of genes during differentiation. Additional suggestive evidence has come from studies of NIH 3T3 cell lines which have been infected with retroviruses expressing the E1A, 13S, 12S, or 9S gene product (109). Expression of the 9S product altered cellular morphology but had no discernible effect on cellular gene expression. In contrast, there was reduced expression of both the collagen and the fibronectin genes in cells containing and expressing the 13S or 12S E1A gene. Given the induction of collagen expression during differentiation of F9 cells, it is tempting to speculate that this results from the relief of negative control by a cellular E1A-like activity in the undifferentiated cells.

These observations also bring into focus the observation concerning control of class I major histocompatibility complex (MHC) gene expression by the adenovirus E1A gene. It was found that there was shutoff of MHC gene expression in adenovirus type 12 (Ad12)-transformed rodent cells but not in cells transformed by Ad5 (113). This observation provided a molecular basis for the biology of adenovirus transformation in which Ad12 more readily forms tumors than does Ad5, possibly owing to the lack of immune recognition as a result of the reduction in MHC expression (41). Further experiments pinpointed the E1A gene as the mediator of this effect; E1A of type 12 in a type 5 virus background produced the shutoff of MHC, whereas an Ad5 E1A in an Ad12 background did not shut off MHC (133). However, the results of recent experiments indicate that the control of the MHC gene may not be analogous to the E1A-mediated repression of the viral enhancers since transcription rates of the MHC gene were the same in Ad5- and Ad12-transformed cells (134). Thus, the negative control in this case appears to be post-transcriptional, the exact nature of which is yet to be established.

TRANSCRIPTION CONTROL AND ONCOGENESIS

Although the focus of much of the work on E1A-mediated transcription control has been to develop a model system of general relevance, it is difficult to forget the effects of E1A expression on cellular phenotype: specifically, that E1A combined with E1B (41) or other cellular oncogenes (110) can induce oncogenic transformation and that E1A alone can immortalize primary cell cultures (54). The question one must ask is, what is the property of E1A that results in this phenotype? With respect to the discussion in this review, is E1A-mediated transcription control involved in transformation? Almost certainly, E1A stimulation of E1B transcription is important in the context of adenovirus transformation. But do the effects go beyond E1B and, in particular, is an alteration of cellular gene expression critical to the process? Several aspects of the basis for positive control of transcription by E1A would support the notion that stimulation of cellular gene expression could be important. First, there is clear evidence that E1A effects activation of transcription through an alteration of cellular transcription factors. Thus, one could suppose that any cellular genes that utilized one of these factors could be stimulated by E1A. Of course, this would only be true if the gene was accessible to the factor and if the concentration of the factor was limiting for that gene. Second, it is quite clear that in some cases there is a stimulation of transcription of cellular genes by E1A. Finally, the best evidence linking E1A-mediated transcription control and oncogenesis is in the F9 teratocarcinoma system. As detailed above, F9 cells are tumorigenic whereas the cells induced to differentiate with retinoic acid and cyclic adenosine 5'-monophosphate are not. Coincident with the oncogenic phenotype is the presence of a cellular E1A-like regulatory activity (58) and, of most significance, the presence of a cellular transcription factor (E2F) known to be regulated by E1A (107).

However, the behavior of certain E1A mutants weighs against the above arguments for a role of positive transcription control in oncogenesis. E1A mutations localized to the 13S specific domain (domain III) or E1A genes expressing only the 12S product no longer *trans* activate with high efficiency but still transform (82, 90, 92). Furthermore, mutations in domain II that have no apparent effect on transcription activation have been found to be transformation negative (82). The former result suggests that transcription activation is not important for transformation, and the latter result clearly demonstrates that an E1A function other than transcription activation must be involved. Indeed, a good candidate function is transcription repression since these two properties, repression and transformation, appear to be tightly linked genetically. Furthermore, the case made for transcription activation in the F9 system can also apply to E1A-mediated transcription repression (40, 51).

However, is the positive control of transcription by E1A clearly ruled out of a role in oncogenesis? For several reasons, the answer is no. First, the genetic analysis has the caveat that the mutants which affect the activation function but still transform do still possess a limited ability to *trans* activate viral gene expression. For instance, several studies have shown that the 12S E1A product can still activate viral transcription, although much less efficiently than the 13S product (28, 78, 147). What is not known is the relative requirement for activation during oncogenesis (if any) as compared with the activation in a lytic infection. Clearly, it could be much less, and if so the limited capacity of the 12S produced might be sufficient. Furthermore, it has now been shown in several cases that the 12S product can activate certain cellular genes with an efficiency nearly equal to that of the 13S product (118, 155).

The results of analyses of mutations in domain II have clearly shown that a function other than *trans* activation is important for transformation. It is thus tempting to speculate that both transcriptional activation and repression of key cellular genes are important for oncogenesis. The difficulty is that in either case this is only a correlation and will remain so until a cellular gene activated or repressed by E1A is shown to be essential for the transformed phenotype. Only then can it be said that either of these processes is important to the process. Such an experiment may be feasible for genes repressed by E1A since, once identified, it would be possible to reintroduce the gene in question into the transformed cell to determine if the phenotype was reversed. In contrast, to prove that a positively regulated gene is critical to the transformed phenotype may be very difficult since it would require a mechanism to turn off the activated gene. An approach with antisense constructs is possible, although by no means is it straightforward.

SUMMARY AND PERSPECTIVES

The study of early adenovirus gene control has now advanced to a detailed understanding of many of the underlying mechanisms. More is known of the processes regulating the expression of this set of genes than for most other

viral systems. In part, this is due to the relative ease of manipulation of the system. However, the major driving force behind these advances has undoubtedly been the relevance of this system of gene control to analogous cellular events. The study of adenovirus gene expression and the regulation of the early adenovirus genes has provided many of the fundamental details in our understanding of eucaryotic gene expression including the identification and isolation of cellular proteins involved in gene control. In this sense, adenovirus does not appear to represent a peculiar or unique situation but rather is an active participant in ongoing cellular events.

The future of this work is bright. Almost certainly, the details of E1A-mediated gene control, including the isolation of the proteins that participate and the manner in which E1A effects the regulation, will be unravelled in the coming few years. Less certain is the elucidation of the role of this system of gene control in the physiology of the cell, with respect to both oncogenesis and normal cellular growth control. Given the importance of such an understanding, there is little doubt that the attainment of these goals will not suffer from a lack of effort and one can therefore anticipate rapid strides in this direction.

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